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Disulfides as Cyanide Antidotes: Evidence for a New In Vivo Oxidative Pathway for Cyanide Detoxification

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It is known that cyanide is converted to thiocyanate in the presence of the enzyme rhodanese. The enzyme is activated by sulfur transfer from an appropriate sulfur donor. The activated enzyme then binds cyanide and transfers the sulfur atom to cyanide to form thiocyanate. This project began as an exploration of the ability of disulfides to act as sulfur donors in the rhodanese-mediated detoxification of cyanide. To our surprise, and contrary to expectations based on efficacy studies in vivo, our in vitro results showed that disulfides are rather poor sulfur donors. The transfer of a sulfur atom from a disulfide to the enzyme must occur via cleavage of a carbon−sulfur bond either of the original disulfide or in a mixed disulfide arising from the reaction of rhodanese with the original disulfide. Extending the reaction time and addition of chloride anion (a nucleophile) did not significantly change the results of the experiment. Using ultrasound as a means of accelerating bond cleavage also had a minimal effect. Those results ruled out cleavage of the carbon−sulfur bond in the original disulfide but did not preclude formation of a mixed disulfide. *S*-Methyl methylthiosulfonate (MTSO) was used to determine whether a mixed disulfide, if formed, would result in transfer of a sulfur atom to rhodanese. While no thiocyanate was formed in the reaction between cyanide and rhodanese exposed to MTSO, NMR analysis revealed that MTSO reacted directly with cyanide anion to form methyl thiocyanate. This result reveals the body's possible use of oxidized disulfides as a first line of defense against cyanide intoxication. The oxidation of disulfides to the corresponding thiosulfinate or thiosulfonate will result in facilitating their reaction with other nucleophiles. The reaction of an oxidized disulfide with a sulfur nucleophile from glutathione could be a plausible origin for the cyanide metabolite 2-aminothiazoline-4-carboxylic acid.

Introduction

Terrorist attacks using cyanide are considered by the U.S. government to have high probability (*1*). While antidotes for cyanide intoxication exist (*2*−*4*), it has yet to be shown that they can be effectively administered in the case of mass exposure. Therefore, there is an urgent need to develop effective cyanide medical countermeasures that can be administered on a large scale.

It is believed that rhodanese, an enzyme found primarily in the mitochondria, is chiefly responsible for the conversion of cyanide to thiocyanate in the body. This sulfur transfer by rhodanese is a classic example of a ping pong mechanism (*5*) and is illustrated in Scheme 1. The first step in the catalytic cycle, as labeled in Scheme 1, is the binding of a sulfur donor to rhodanese. In the second step, the sulfur donor transfers a sulfur atom to cysteine-247. This gives rise to persulfido-cysteine-247 (*6*−*8*), the active form of rhodanese. The third step in Scheme 1 is the binding of hydrogen cyanide (HCN) to the activated enzyme (*9*). This is followed by the transfer of a sulfur atom from the activated enzyme to the bound HCN in step four resulting in the formation of thiocyanic acid (HSCN). The fifth and final step is the release of HSCN from the enzyme. Under physiological conditions, released HSCN is converted to the thiocyanate anion. Because thiocyanate salts have an oral LD_{50} more than 2 orders of magnitude greater than the oral LD_{50} value of potassium cyanide in mice and rats, this is clearly a detoxification mechanism.

Historically, oxidized sulfur species such as sodium thiosulfate, ethyl thiosulfonate, 2-aminoethyl thiosulfonate, and others have been shown to be the most effective *in vitro* sulfur donors for rhodanese (*2*−*4*). Sulfur donors such as disulfides [including bis(4-methoxyphenyl) disulfide (WR2467) and diallyl disulfide (DADS) (Table 1), which is found in garlic] have been found to be effective as in vivo therapeutic agents for cyanide intoxication (*10, 11*). It is believed that the efficacy of these sulfur-containing compounds also arises from their ability to transfer a sulfur atom to rhodanese.

The mechanism of sulfur transfer from disulfides to rhodanese has yet to be elucidated. Two possible mechanisms for the activation of rhodanese by a disulfide are posited in Scheme 2. In mechanism A, reaction of a disulfide with a nucleophile cleaves the carbon−sulfur bond forming an alkyl persulfide (A) and the alkylated nucleophile. The alkyl persulfide reacts directly with rhodanese to form the activated form of rhodanese and an alkyl sulfide (RSH). Mechanism B involves direct reaction of the enzyme with a dialkyl disulfide to form a mixed disulfide. This mixed disulfide reacts with a nucleophile to generate the activated form of the enzyme. We set out to determine experimentally which mechanism explains the activation of rhodanese by disulfides.

The direct transfer of a sulfur atom to rhodanese, such as seen with sodium thiosulfate, is more efficient than either two-

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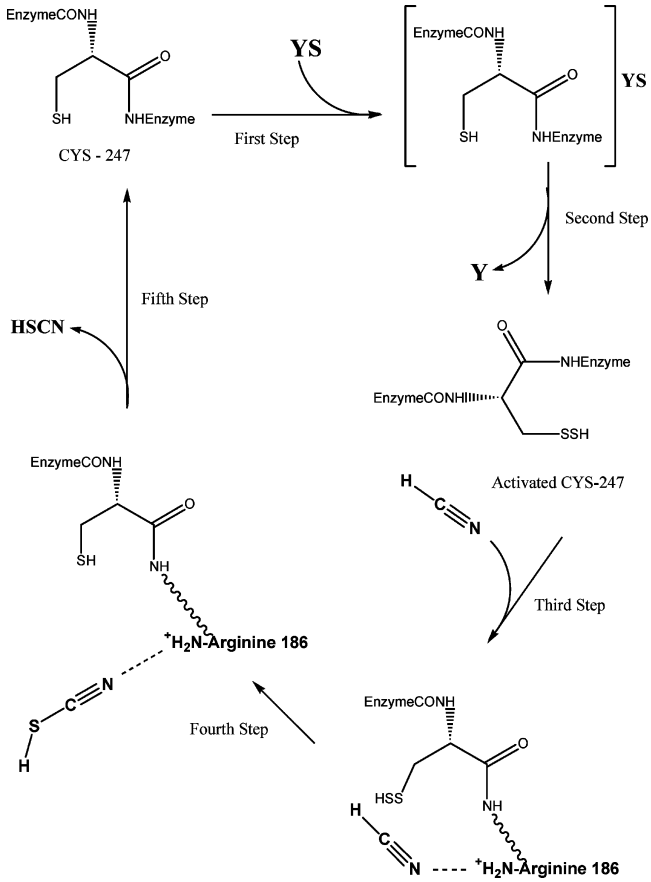
Scheme 1. Mechanism of Rhodanese-Mediated Detoxification of Cyanide[a]



[a] The catalytic cycle is initiated when a sulfur donor, designated YS, binds to rhodanese. The sulfur donor transfers a sulfur atom to cysteine 247, forming persulfido cysteine. This transfer activates the enzyme. The depleted sulfur donor, Y, is released from the enzyme. The activated enzyme binds HCN via a hydrogen bond with a positively charged residue. We have arbitrarily chosen ARG-186, a residue in the active site for sulfur transfer, as that residue. After binding, rhodanese transfers the terminal sulfur of the persulfido cysteine to HCN to form HSCN. Thiocyanate is released, and the enzyme returns to its initial state.

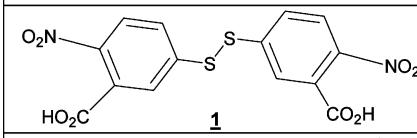
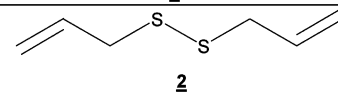
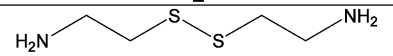
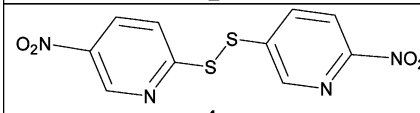
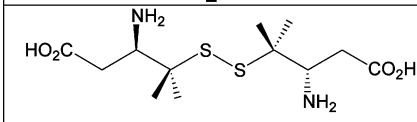
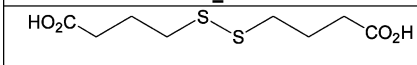
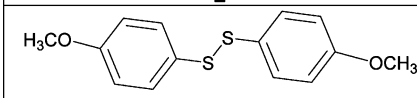
step process. Nonetheless, a series of studies have presented evidence that disulfides can be effective antidotes for cyanide intoxication (*10*, *11*). In one study, the compound WR2467 (compound **7**, Table 1), was reported to be effective as an in vivo cyanide antidote in mice (*11*). It is especially difficult to envision nucleophilic cleavage of the carbon−sulfur bond in WR2467 as it is well-known that nucleophilic cleavage of an aromatic carbon−sulfur bond does not occur except under unusual circumstances.

This work focuses on rationalizing the poor in vitro efficacy of disulfides with the widely held belief that disulfides can act as sulfur donors in vivo. The results of these studies will not only demonstrate that disulfides play no role in the enzyme-mediated detoxification of cyanide but are oxidized to the corresponding thiosulfonate, which act as cyanide scavengers.

Materials and Methods

All compounds used in this study were either purchased directly from Sigma Aldrich or were obtained from internal supplies. Deionized water was produced by a Millipore Direct-Q 3UV system (Millipore Inc., Billerica, MA) and had a resistance of 18 MΩ. pH readings were obtained using an Orion 3-star pH Benchtop (ThermoElectron Corp., Beverly, MA). Sonication was performed

Table 1. Disulfides Tested for Efficacy as Sulfur Donors for Rhodanese[a]

Sulfur donor	E
Thiosulfate (standard)	1.0
 **1**	0.005
 **2**	0.007
 **3**	0.005
 **4**	0.012
 **5**	0.016
 **6**	0.008
 **7 or WR2467**	0.007

[a] The column labeled "E" is the performance of the disulfide relative to the performance of an equimolar amount of thiosulfate. E was calculated by dividing the amount of thiocyanate produced by the sulfur donor by the amount of thiocyanate produced using thiosulfate over a 5 min period.

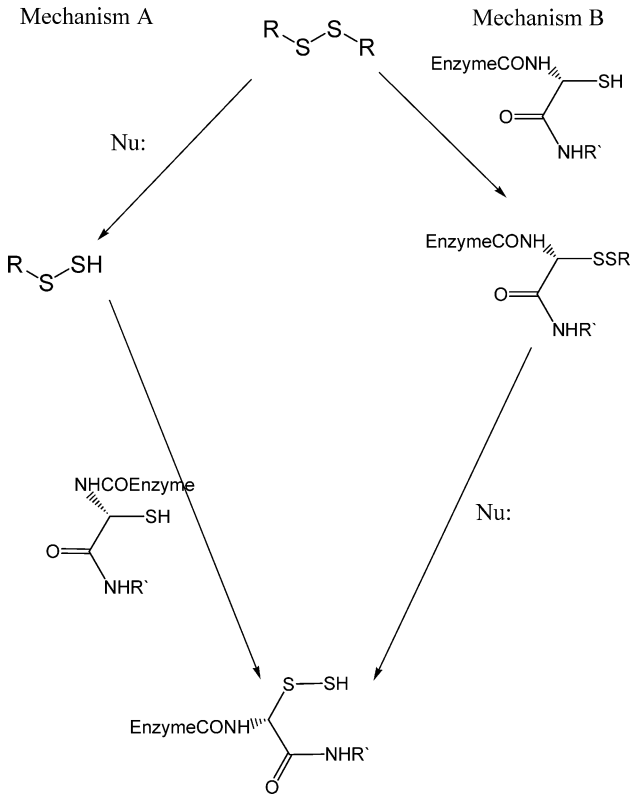
using a Bransonic Ultrasonic Cleaner model 2510R-MT (Branson Ultrasonics Corp., Danbury, CT). Absorbance of the iron−thiocyanate complex was measured on a Beckman DU-7 spectrophotometer (Beckmann Instruments, Palo Alto, CA). A 10 mM concentration of phosphate buffer at pH 7.4 was used throughout. All assays were conducted in triplicate.

Measurement of the Rhodanese-Mediated Conversion of Cyanide to Thiocyanate To Determine the Efficacy of Sulfur Donors. This assay was an adaptation of the original procedure of Wang and Volini (*12*). The assay mixture was composed of 50 μL of 100 mM potassium cyanide solution, 50 μL of 100 mM solution of sulfur donor, and 50 μL of rhodanese (3.5 U/mL) diluted with 700 μL of 10 mM phosphate buffer at pH 7.4. Sulfur donors insoluble in phosphate buffer were dissolved in dioxane to make solutions that were 100 mM in disulfide. After 5 min in a 37 °C water bath, the enzymatic reaction was quenched with 0.5 mL of 14.8% aqueous formaldehyde solution. This was followed by the addition of 1 mL of 165 mM iron(III) nitrate solution in 9.3% nitric acid to produce the highly colored iron(III) thiocyanate complex. The absorbance of this complex was measured at 458 nm. All blanks were prepared in the same manner, with the enzyme aliquot replaced by an equivalent volume of buffer.

Determination of the Efficacy of Sulfur Donors Relative to Sodium Thiosulfate. The efficacy for each sulfur donor was computed by dividing the value of the average absorbance for the assay of the sulfur donor run in triplicate by the average absorbance for an identical experiment using sodium thiosulfate.

Determination of Unreacted Cyanide after Use of *S*-Methyl Methylthiosulfonate (MTSO) as a Sulfur Donor for the Rhodanese-Mediated Conversion of Cyanide to Thiocyanate. This assay was run after using MTSO to form a mixed disulfide with

Scheme 2. Potential Mechanisms for Disulfide Activation of Rhodanese[a]



[a] Mechanism A shows the reaction of a disulfide with a nucleophile, cleaving the carbon−sulfur bond, thus forming a persulfide intermediate. The alkyl persulfide transfers a sulfur atom to rhodanese to generate the activated form of rhodanese. Mechanism B shows the reaction of rhodanese with a disulfide (RSSR) to form a mixed disulfide. This intermediate reacts with a nucleophile, cleaving the "R" group to form the activated form of the enzyme.

rhodanese. Because the initial assay showed no production of thiocyanate, it was necessary to determine if there was any cyanide left in the assay mixture. The assay to detect unreacted cyanide involved the use of 4,4′-trisulfane-1, 3-diyldibutane-1-sulfinic acid [ICD 1982 in SMILES notation OS(═O)CCCCSSSCCCCS(═O)O]. Unpublished results from our lab have demonstrated that this trisulfide reacts with cyanide to produce thiocyanate without the need for rhodanese to mediate the reaction. After the initial assay with MTSO had run 5 min, 50 μL of a 100 mM aqueous solution of ICD 1982 was added. After an additional 5 min, the assay mixture was quenched with formaldehyde; iron(III) nitrate was added as described previously. The absorbance of the solution was again measured at 458 nm.

Determination of the Product from the Reaction between MTSO and KCN. An assay was needed to identify the product from the reaction between MTSO and cyanide. A Varian 600 MHz NMR spectrometer was employed to follow this direct reaction. An NMR sample was prepared by dissolving 0.25 mmol of MTSO in 0.5 mL of pH 7.4 buffer. To this solution was added 50 μL of D_2O to provide a lock signal. After the ^{13}C and ^{1}H NMR spectra of the thiosulfonate were obtained, the NMR sample was emptied into a vial containing 0.24 mmol of ^{13}C-labeled KCN. After dissolution of the cyanide salt, a second NMR spectrum was taken to monitor the progress of product formation. The time between spectra was approximately 1 min.

The preliminary identification of the new compound via NMR was confirmed by mass spectral analysis. The NMR sample was transferred to a 2 mL sample vial. Five hundred microliters of ethyl acetate (EtOAc) was added to the vial, and it was vortexed for 1 min. The top layer (EtOAc) was withdrawn and placed into a second sample vial. Ten microliters of this extract was diluted into 1.0 mL of EtOAc in a third sample vial and was analyzed by gas chromatography/mass spectrometry (GC/MS) as described below.

GC/MS was conducted on an Agilent Technologies (Santa Clara, CA) 6890 gas chromatograph interfaced to an Agilent 5973 mass-selective detector. The GC was fitted with a 30 m × 0.25 mm i.d. DB-5 ms bonded phase capillary column, 0.25 μm film thickness manufactured by Agilent Technologies. Helium was used as the carrier gas in the constant flow mode at 0.8 mL/min^{-1}. The oven temperature was held initially at 35 °C for 5 min, programmed from 35 to 280 at 50 °C min^{-1}, and held at 280 °C for 3 min. Splitless injections of 1 μL were made using an Agilent 7683 autosampler. The injection port temperature was set to 250 °C, the purge time to 3 min, and the transfer line temperature to 265 °C. MS detection was accomplished in the EI+ mode with a quadrupole temperature of 150 °C and an ion source temperature of 230 °C. Data were acquired in scan mode ranging from 35 to 200 amu. The spectra were averaged across the peak at half height and background subtracted by scans immediately following the peak. The resulting spectrum was compared to the NIST (2002) spectral library entry for HSCN, methyl ester (CAS 556-64-9).

Quantum Mechanical Calculations To Determine the Stability of a Vicinal Disulfoxide Relative to a Thiosulfonate. Quantum mechanical calculations were performed to determine the preferred outcome of disulfide oxidation. Geometry optimizations were carried out at the MPW1PW91/6-311+g(2d,p) level of theory (*13*) using the Gaussian G03, Revision E.01 program (*14*) as implemented at the Alabama Supercomputer Center in Huntsville, AL. This was followed by a frequency calculation at the same level of theory to validate that the optimized structures were minima and not maxima on the potential energy surface. The wave function of each optimized structure was processed by the AIM2000 program of Biegler-Koenig (*15*) to assess the relative strength of the sulfur−sulfur bond in each molecule.

Results

The amount of thiocyanate produced in the rhodanese reaction with each disulfide relative to an equimolar amount of sodium thiosulfate over a 5 min period is listed in Table 1. Of particular note is the fact that neither the aliphatic nor the aromatic disulfides generated a significant amount of thiocyanate in comparison to sodium thiosulfate. These results are surprising since they are contrary to the expectation based on the reported in vivo potency of compounds **2** (DADS) and **7** as cyanide antidotes in mice and rats (*16, 17*). To explore the discrepancy between in vitro and in vivo efficacy, DADS was selected. Second, DADS has two allyl groups. An allyl group can be readily cleaved via nucleophilic displacement reactions; therefore, there should be a low energetic barrier for producing activated rhodanese. To test the hypothesis that disulfides activate rhodanese via a nucleophile-based mechanism, several experiments were conducted with DADS.

Increasing the reaction time of the assay from 5 to 15 min caused an increase in the total amount of thiocyanate produced. However, that amount was nearly 2 orders of magnitude less than that produced by thiosulfate in a 5 min period. Those experiments were repeated, sonicating the assay mixture containing the DADS to enhance the rate of reactions (*18, 19*). Table 2 summarizes the results of the sonication experiments. While tripling the in vitro reaction time resulted in a near tripling of the amount of product formed, in the end, only one percent of the cyanide was converted to thiocyanate, less than 2% of the amount of thiocyanate formed when sodium thiosulfate is the sulfur donor. This result does little to explain the in vivo potency of DADS. If these results arise from cleavage of an allyl group, then this cleavage is a slow process. The results militate against the mechanisms of activation shown in Scheme

Table 2. Effect of Time and Sonication on the Production of Thiocyanate from Cyanide Using DADS and Rhodanese[a]

	no sonication		with sonication	
time	5 min	15 min	5 min	15 min
% conversion	0.41	1.10	0.72	1.50
	0.46	1.25	0.67	1.29
	0.41	1.19	0.91	1.45
average % conversion	0.42	1.17	0.77	1.41

[a] Percent conversion is calculated as the amount of thiocyanate divided by the initial amount of cyanide in the assay.

2 since a slow activation step would mean an overall slow and therefore inefficient process of converting cyanide to thiocyanate.

To determine if nucleophilic substitution would eventually lead to activation of rhodanese by a disulfide sulfur donor, a standard assay was run with DADS and 1.5 mol equiv of sodium chloride relative to the amount of DADS in the assay. Chloride was chosen as it is known to be a good nucleophile that is also ubiquitous in vivo. In the presence of chloride, less than 5% of the cyanide had been converted to thiocyanate, less than 10% of the amount converted by sodium thiosulfate. Repeating this experiment with sonication again resulted in less than a 5% conversion of cyanide into thiocyanate.

While this result casts doubt on the hypothesis that disulfides can activate rhodanese, it remains possible that a reaction occurs between cyanide and DADS. A direct reaction between cyanide and DADS would result in the formation of allyl thiocyanate and allyl sulfide. If this were the case, and if, indeed, cleavage of the allyl−sulfur bond were slow, formation of allyl thiocyanate would be dominant, leaving small amounts of cyanide for the enzymatic reaction. Therefore, one would expect meager conversion of cyanide into thiocyanate.

To evaluate this possibility, an experiment was devised to determine the amount of cyanide consumed in the presence of DADS and rhodanese. A solution of rhodanese, DADS, and cyanide in pH 7.4 phosphate buffer was allowed to react for 5 min followed by the addition of ICD1982. This compound is a trisulfide, which has been shown to convert cyanide directly into thiocyanate (*25*). Stopping the reaction 5 min after the addition of the trisulfide resulted in a nearly quantitative conversion of cyanide into thiocyanate. This near quantitative conversion demonstrated that there was no reaction between cyanide and DADS. Repeating this experiment while sonicating the reaction mixture prior to introduction of cyanide produced the same result. Had the sulfur−carbon bond in DADS been cleaved, the resulting persulfide would have readily reacted with rhodanese. Because minimal amounts of thiocyanate were formed, the persulfide was not being produced. However, it was unclear whether a mixed disulfide was ever formed (mechanism B). If formation of a mixed disulfide could be demonstrated and there was still insignificant production of thiocyanate, then it could be reasonably concluded that neither mechanism A nor mechanism B can account for disulfide activation of rhodanese. Therefore, it was necessary to rule out the formation of a disulfide from the direct reaction of a disulfide and the sulfhydryl group in the active site of the enzyme.

To rule out the formation of a disulfide from the direct reaction of a disulfide and the sulfhydryl group in the active site of the enzyme, MTSO was reacted directly with rhodanese. It is known that MTSO will react with sulfhydryl groups within enzymes to form disulfides akin to that shown in mechanism B in Scheme 2. The exposure of rhodanese to MTSO followed by the introduction of cyanide produced no detectable thiocyanate in the reaction mixture. The addition of ICD1982 to the

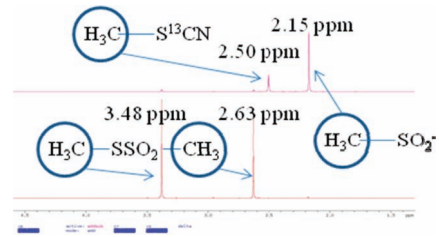


Figure 1. ^{1}H NMR spectra of *S*-methyl methanethiosulfonate (MTSO) and reaction mixture of the thiosulfonate with K^{13}CN. The lower spectrum is that of MTSO alone, while the upper spectrum is that of the reaction mixture between MTSO and K^{13}CN. The peaks in each spectrum are identified in the graphic above. The signal for the methyl group of methyl thiocyanate at 2.5 ppm is a doublet arising from long-range splitting between the methyl protons and the labeled ^{13}C-labeled carbon of the thiocyanate (SCN) group. The byproduct of the reaction between cyanide and MTSO produced the signal at 2.15 ppm.

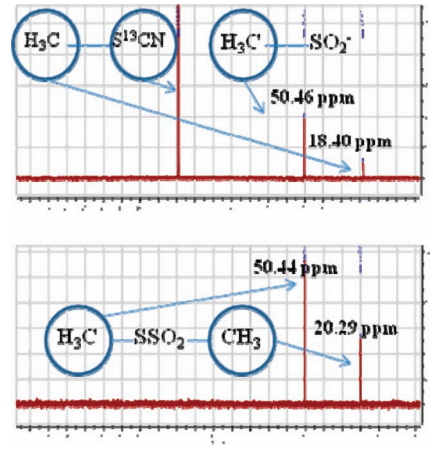


Figure 2. ^{13}C NMR spectra of MTSO and reaction mixture of MTSO with K^{13}CN. The lower spectrum is that of MTSO; the upper spectrum is that of the reaction mixture between MTSO and K^{13}CN. The peak in the upper spectrum at 119.07 ppm is the peak corresponding to the carbon in the SCN moiety of CH_3SCN. The peak at 18.4 ppm corresponds to the methyl group. The peak at 50.46 ppm in the upper spectrum is the reaction product from MTSO.

reaction mixture produced no additional thiocyanate. It is clear that cyanide reacted directly with MTSO, precluding any formation of thiocyanate.

To determine how MTSO reacted with cyanide, an equimolar mixture of MTSO and ^{13}C-labeled potassium cyanide was studied using NMR spectroscopy. The ^{1}H NMR spectrum of this mixture, obtained less than 5 min after the reactants were mixed, is shown in Figure 1. The ^{1}H NMR spectrum of the reaction mixture is markedly different from that of MTSO (KCN has no detectable signal in the proton region). The spectrum of the starting material has two singlets, resulting from two different, noninteracting methyl groups, identified in the lower spectrum in Figure 1. The spectrum of the reaction mixture, the upper one in Figure 1, shows that the signals belonging to MTSO have almost disappeared. Two new signals, a doublet centered at 2.6 ppm and a singlet at 2.17 ppm, have appeared. The appearance of a doublet means that this new compound has another NMR-active nucleus interacting with one of the methyl groups. We hypothesized that this doublet arose via the interaction of ^{13}CN with one of the methyl groups from MTSO.

Two ^{13}C NMR spectra, one of the reaction mixtures (upper), the other of MTSO (lower), are displayed in Figure 2. The comparison of the ^{13}C spectra gives a clue for identifying the products of the reaction. In the upper spectrum, no signal for K^{13}CN was observed, bolstering the hypothesis that MTSO has reacted directly with cyanide. Therefore, the spectrum is a

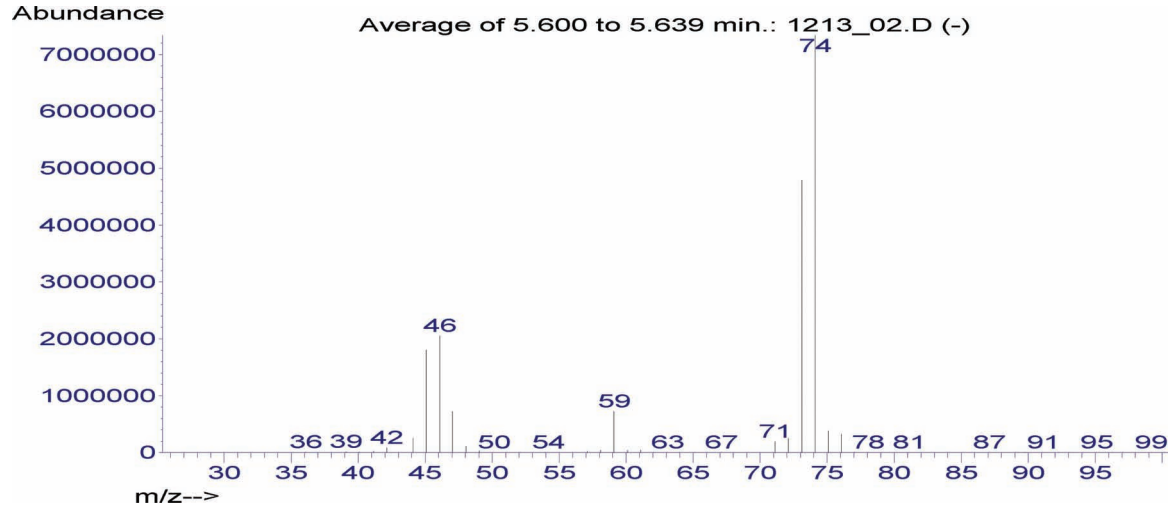


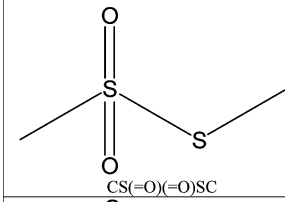
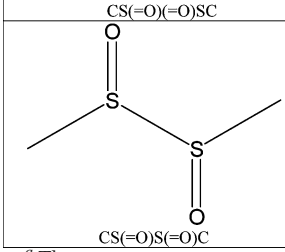
Figure 3. MS spectrum of NMR reaction mixture described in the Results section. The *m*/*e* peak of 74 corresponds to $CH_3S^{13}CN$.

mixture of two compounds. The furthest downfield peak is at 119.07 ppm. This chemical shift tentatively identifies the carbon as being in a thiocyanate moiety. The signal at 18.40 ppm is assigned as CH_3-S since it is extremely close to the $S-CH_3$ signal at 20.29 ppm in the MTSO spectrum. The peak at 50.46 ppm in the product spectrum most likely corresponds to the $CH_3SO_2^-$ ion, the result of cleavage of the sulfur−sulfur bond in MTSO. Therefore, the reaction products were tentatively identified as $CH_3S^{13}CN$ and $CH_3SO_2^-$. To unambiguously identify the organic reaction product, we employed mass spectrometry (MS).

The MS spectrum for the reaction product shown in Figure 3 displays one major peak, with an *m*/*e* of 74; this is the expected parent ion for ^{13}C labeled methyl thiocyanate. This unambiguously identifies the product as $CH_3S^{13}CN$. With this identification, the product peak at 50.46 can be assigned to $CH_3SO_2^-K^+$, the compound resulting from the cleavage of the sulfur−sulfur bond in MTSO.

It has been reported in the literature (*17*) that disulfides can be readily oxidized in vivo. Oxidation of a disulfide would first result in formation of an alkyl thiosulfinate. Further oxidation would lead to the corresponding thiosulfonate, although oxidation could yield a vicinal disulfoxide. Because both are possible, the question arises as to which compound would be more stable, the thiosulfonate or the vicinal disulfoxide. To answer that question, we employed quantum mechanical calculations to determine the minimum energy structure for both compounds. The results of these calculations are summarized in Table 3. Because the two compounds are merely structural isomers, the lower energy structure would be the thermodynamically favored structure. The difference in structures is striking with the vicinal disulfoxide having a sulfur−sulfur bond length of 0.3 Å greater than that in the thiosulfonate. This has the concomitant result that the electron density at the bond critical point for the sulfur−sulfur bond in the thiosulfonate is nearly 50% greater than at the same bond critical point in the disulfoxide. The results also show that the thiosulfonate structure is favored over the vicinal disulfoxide by nearly 20 kcal. These results are in line with an earlier report of calculations done at a lower level of theory on vicinal disulfoxides and thiosulfonates (*20*). The results of these calculations are in line with the reported chemistry of vicinal sulfoxides (*23*). It has been well-established that should oxidation of a disulfide form a vicinal disulfoxide, there would be a subsequent and rapid molecular rearrangement to form the thiosulfonate (*24*).

Table 3. Key Data from Quantum Mechanical Calculations on MTSO and Dimethyl Disulfoxide[a]

Compound	Energy	ΔG	r(S-S)	ρ(S-S)
 CS(=O)(=O)SC	-1026.65242	0.0	2.104	0.343
 CS(=O)S(=O)C	-1026.61989	20.4	2.325	0.226

[a] The two structures are shown with their respective SMILES representation written underneath. Energy is in units of Hartrees. The change in energy (Δ*E*) is relative to the thiosulfonate and is in units of kcal/mol. The column labeled "r(S-S)" shows the internuclear separation of the vicinal sulfur atoms in units of Angstroms. The column labeled "ρ(S-S)" is the electron density at the sulfur−sulfur bond critical point, a measure of the strength of the two bonds.

Discussion

The major conclusion from this work is that disulfides do not activate rhodanese in vitro. DADS, compound **2** (Table 1), a very good model to evaluate the role of nucleophilic displacement in disulfide activation of rhodanese, generated extremely small amounts of thiocyanate. Attempts using sonication and addition of chloride ions to facilitate carbon−sulfur bond cleavage did not increase the thiocyanate yield. In short, it is apparent that neither of the nucleophilic mechanisms proposed in Scheme 2 is operative.

Nevertheless, some disulfides can act in vivo as effective medical countermeasures against cyanide intoxication. One can envision several explanations for this apparent discrepancy. It is possible that an unknown cofactor exists in vivo that we did not supply in our in vitro system, although there is no evidence that rhodanese requires any cofactors for activation other than a substrate that transfer a sulfur atom to the enzyme. A second possibility would be a direct reaction between cyanide and disulfide, but that reaction has been reported to be slow (*21*), and those results were confirmed in this study; very little cyanide

was consumed by DADS. Certainly, the direct reaction is too slow to act as a significant in vivo detoxification mechanism. Perhaps the most likely alternative explanation is that disulfides are metabolized in vivo to compounds that react quickly and directly with cyanide and thus are able to act as cyanide scavengers. The reaction of MTSO, an alkyl thiosulfonate, with cyanide is rapid and direct and does not require the intermediacy of an enzyme. If similar compounds were produced in the body by metabolism of disulfides, they could account for the in vivo effectiveness of disulfides as cyanide antidotes. It has been shown that diallyl disulfide can be oxidized in vitro to its corresponding thiosulfinate allicin (*17*), which could be further oxidized to the thiosulfonate, as shown in the computational results presented earlier. We conclude that, probably, disulfides do not detoxify cyanide by activating rhodanese; rather, we propose that they are metabolically oxidized to the corresponding thiosulfonates, which scavenge cyanide from both the cells and the bloodstream.

This conjecture is supported by the production of the cyanide metabolite 2-aminothiazoline-4-carboxylic acid (ATCA) detected by Wood and Cooley in their investigation of the detoxification of cyanide by cystine (*22*): ATCA appearing as a result of intermolecular cyclization of the cysteine thiocyanate. The formation of this cyanide metabolite likely arises from the reaction of cyanide with thiosulfonate of glutathione disulfide and is evidence that in vitro-generated thiosulfonates will react with cyanide.

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